REMARKS

Claims 1, 5, 9, 12, 53, 54, 60, and 61 are pending.

Rejections under 35 USC § 103

Claims 1, 5, 9, 12, 53, 54, 60, and 61 are rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Varner [United States Patent Number (USPN) 7,311,911] in view of Li et al. (United States Publication Serial Number 2004/0048312). In view of Applicant's arguments presented herein, the rejection, as it applied to claims 1, 5, 9, 12, 53, 54, 60, and 61, is respectfully traversed.

The claims are directed to a method for disrupting survival signaling from a bone marrow microenvironment to single breast cancer cells or breast cancer cell micrometastases in a mammal with breast cancer, said method comprising administering to said mammal with breast cancer (claim 1) or a method of inhibiting cellular proliferation or inducing cell death or cellular differentiation of single breast cancer cells or breast cancer cell micrometastases in a mammal with breast cancer or for treating a single breast cancer cell or breast cancer micrometastases in a mammal with breast cancer comprising administering to the mammal with breast cancer (claim 12), either of said methods comprising administering as adjuvant therapy an agent effective in blocking the interaction of an integrin with an extracellular matrix protein of the bone marrow microenvironment, wherein the integrin is alpha 5 beta 1 and the extracellular matrix protein is fibronectin. As previously asserted, the present method is directed to breast cancer patients who have no detectable disease as a result of previous treatment. Such patients are treated with adjuvant therapy to prevent relapse of disease. See, for example, paragraphs [0004], [0006], [0008], and [0079] of the instant specification for additional details.

The Office Action recognizes that Varner does not teach or suggest an adjuvant therapy, but relies on Li et al. for allegedly providing such teaching. Li et al. is directed in its entirety to αvβ6 integrin and its role in human cancers and a monoclonal antibody (mBLA3) specific for αvβ6 integrin. See entire document and the Abstract, for example.

For the record, Applicant asserts that the present inventor assessed and did not observe an increase in the expression of either αv or β6 in the dormant (growth arrested)

breast cancer cells analyzed in accordance with the present invention. For the purposes of review and as detailed in the present specification at, for example, paragraph [0027] of United States Publication Serial Number 2006/0035825,

> *"Breast cancer cells that metastasize to the bone marrow are arrested by deposits of FGF-2 in the bone marrow stroma. FGF-2 induces overexpression of integrins α5 and β1 and leads to massive cell death through unligated or inappropriately ligated integrins by a process termed integrin-mediated death (IMD). Adherence and appropriate ligation of α5β1 on the surviving cells by fibronectin in the stroma interrupts IMD and initiates survival signaling through PI3K. This results in dormancy of the non-cycling sells and protection from cell death induced by cytotoxins. Disruption of the integrin-fibronectin interaction would discontinue survival signaling and initiate IMD. This would render the metastatic cells sensitive to chemotherapy."* Emphasis added.

The findings of the present specification demonstrate that ligation of α5β1 integrin enables dormant breast cancer cells to escape IMD resulting from FGF-2 mediated overexpression of integrins α5 and β1 and thus, teach that α5β1 integrin plays a particular role in the ability of dormant breast cancer cells to survive in the bone marrow microenvironment. The present inventor's results also reveal that αvβ6 integrin, which is not upregulated in breast cancer cells in the bone marrow microenvironment, does not appear to play a role in survival of growth arrested breast cancer cells metastasized to the bone marrow.

Moreover, a review of the literature reveals that there is a general consensus that αvβ6 integrin increases with carcinogenesis in most primary tumors and some metastases, but primarily in squamous cell carcinomas and colon and ovarian epithelial cells. Expression of αvβ6 integrin appears to increase with cell crowding. The literature is, furthermore, silent with regard to expression αvβ6 integrin in metastatic breast cancer. As detailed in the Van Aarsen et al. paper (Cancer Research 68:561, 2008; attached hereto), a minority of primary breast carcinomas only express this fibronectin binding integrin.

In summary, therefore, there is no significant role attributed to αvβ6 integrin in primary breast tumors, no evidence of αvβ6 integrin expression in metastatic breast cancer cells, and no evidence of an increase in αvβ6 integrin expression in dormant breast cancer cells. In light of the above, the findings of the present inventor and general knowledge in the field teach away from there being any relationship between the expression and/or activity of αvβ6 integrin and survival of dormant micrometastatic breast cancer cells in the bone marrow. That being the case, Applicant asserts that there is no motivation to combine the teachings of Varner with those of Li et al. because these references are directed to different integrins that have divergent expression patterns and activities and general knowledge in the field does not support the existence of a nexus with respect to these integrins with regard to dormant micrometastatic breast cancer cells in the bone marrow.

Indeed, the absolute reliance on Li et al. for suggesting adjuvant therapy (which is mentioned only once in passing), overlooks the fact that there are dozens of integrins with many overlapping and disparate ligands. In other words, it is understood in the art that not all integrins are alike and, more to the point, serve varied and distinctly different functions in different settings. Simply teaching the use of antibodies to a particular integrin in an adjuvant setting does not extend to the use of antibodies to any other integrin in any adjuvant setting. An ordinarily skilled practitioner would appreciate that in the absence of an understanding as to which integrin or integrins are relevant in a particular clinical setting with respect to expression and activity, there is no way to predict with a reasonable expectation of success that use of antibodies to a particular integrin will have any clinical utility.

The comments of the Office Action pertaining to Varner allegedly teaching that administration to a subject can either be over a relatively short period of time or can be over a more prolonged period of time and that different therapeutic protocols can be used to achieve the most effective regime are duly noted. The Examiner is, however, respectfully reminded that Varner is invariably directed to a method for reducing or inhibiting angiogenesis for therapeutic purposes. With regard to cancer, the intended therapeutic purpose as taught throughout Varner is to reduce or inhibit tumor angiogenesis. An ordinarily skilled practitioner would appreciate that such a therapeutic

purpose only pertains to a patient with detectable disease (i.e., a detectable tumor or tumors) and falls outside an adjuvant setting.

In light of the above, Applicant asserts that an ordinarily skilled practitioner would not be motivated to combine the teachings of Varner and Li et al. to allegedly arrive at the present invention because general knowledge in the field does not support the existence of a nexus between expression and/or activity of α5β1 integrin and αvβ6 integrin with regard, in particular, to dormant micrometastatic breast cancer cells in the bone marrow. The present inventor's determination that αvβ6 integrin expression is not upregulated in dormant micrometastatic breast cancer cells, whereas α5β1 integrin expression is upregulated and confers enhanced survival, confirms and corroborates the prevailing scientific thought that these are dramatically disparate integrins with dissimilar expression patterns and activities. In view of the above, an ordinarily skilled practitioner would have no motivation to combine the teachings of Varner and Li et al.

In view of the above, Applicant deferentially requests that the rejection of claims 1, 5, 9, 12, 53, 54, 60, and 61 under 35 U.S.C. § 103(a) as allegedly unpatentable over Varner and Li et al. be reconsidered and withdrawn.

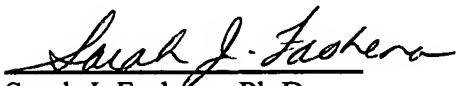
Fees

No additional fees are believed to be necessitated by this amendment. However, should this be an error, authorization is hereby given to charge Deposit Account No. 11-1153 for any underpayment or to credit any overpayment.

Conclusion

It is submitted, therefore, that the claims are in condition for allowance. No new matter has been introduced. From the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order, and such action is earnestly solicited. In the event that there are any questions concerning this amendment, or application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Respectfully submitted,



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Enclosures: Petition for a One Month Extension of Time
Request for Continued Examination
Van Aarsen et al. (Cancer Research 68:561, 2008)

Antibody-Mediated Blockade of Integrin $\alpha_v\beta_6$ Inhibits Tumor Progression *In vivo* by a Transforming Growth Factor-β–Regulated Mechanism

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Abstract

The $\alpha_v\beta_6$ integrin is up-regulated on epithelial malignancies and has been implicated in various aspects of cancer progression. Immunohistochemical analysis of $\alpha_v\beta_6$ expression in 10 human tumor types showed increased expression relative to normal tissues. Squamous carcinomas of the cervix, skin, esophagus, and head and neck exhibited the highest frequency of expression, with positive immunostaining in 92% (n = 46), 84% (n = 49), 68% (n = 56), and 64% (n = 100) of cases, respectively. We studied the role of $\alpha_v\beta_6$ in Detroit 562 human pharyngeal carcinoma cells *in vitro* and *in vivo*. Prominent $\alpha_v\beta_6$ expression was detected on tumor xenografts at the tumor-stroma interface resembling the expression on human head and neck carcinomas. Nonetheless, coculturing cells *in vitro* with matrix proteins did not up-regulate $\alpha_v\beta_6$ expression. Detroit 562 cells showed $\alpha_v\beta_6$-dependent adhesion and activation of transforming growth factor-β (TGF-β) that was inhibited >90% with an $\alpha_v\beta_6$ blocking antibody, 6.3G9. Although both recombinant soluble TGF-β receptor type-II (rsTGF-βRII-Fc) and 6.3G9 inhibited TGF-β–mediated Smad2/3 phosphorylation *in vitro*, there was no effect on proliferation. Conversely, *in vivo*, 6.3G9 and rsTGF-βRII-Fc inhibited xenograft tumor growth by 50% (n = 10, P < 0.05) and >90% (n = 10, P < 0.001), respectively, suggesting a role for the microenvironment in this response. However, stromal collagen and smooth muscle actin content in xenograft sections were unchanged with treatments. Although further studies are required to consolidate *in vitro* and *in vivo* results and define the mechanisms of tumor inhibition by $\alpha_v\beta_6$ antibodies, our findings support a role for $\alpha_v\beta_6$ in human cancer and underscore the therapeutic potential of function blocking $\alpha_v\beta_6$ antibodies. [Cancer Res 2008;68(2):561–70]

Introduction

The $\alpha_v\beta_6$ receptor is one member of the large family of integrin proteins that are expressed in heterodimeric form on the surface of essentially all cells (1, 2). Integrins are involved in regulating a variety of cellular processes, including adhesion, migration, proliferation, differentiation, and death (3–5). The expression of $\alpha_v\beta_6$ is restricted primarily to epithelial cells where it is expressed at relatively low levels in healthy tissue and significantly up-regulated during development, injury, wound healing, and in epithelial tumors (6–11). The $\alpha_v\beta_6$ integrin may have multiple regulatory functions in tumor cell biology. The extracellular and transmembrane domains have been shown to mediate transforming growth factor-β (TGF-β) activation and adhesion (12, 13). The cytoplasmic domain of the β_6 subunit contains a unique 11-amino-acid sequence that has been implicated in mediating cell proliferation, matrix metalloproteinase production, invasion, and survival (14–18).

$\alpha_v\beta_6$ can bind to several ligands, including fibronectin, tenascin, and the latency-associated peptide-1 and latency-associated peptide-3 (LAP1 and LAP3), the NH_2-terminal region of the latent precursor form of TGF-β1 and TGF-β3, through a direct interaction with an RGD motif (2, 13, 19). $\alpha_v\beta_6$ binding to LAP1 or LAP3 leads to activation of the latent precursor form of TGF-β1 and TGF-β3 (13). Thus, up-regulated expression of $\alpha_v\beta_6$ can lead to local activation of TGF-β, which then has the potential to affect neighboring cells in a paracrine fashion. Up-regulated $\alpha_v\beta_6$ expression has been shown to be associated with increased epithelial to mesenchymal transition, increased tumor cell invasion, and increased metastatic potential *in vivo* (9, 16, 20, 21). The overexpression of $\alpha_v\beta_6$ on tumors, particularly at the tumor-stromal interface, may reflect a unique mechanism for local activation of TGF-β1 and the ability to promote tumor progression, invasion, and metastasis.

The TGF-β1 cytokine is a pleiotropic growth factor that can regulate cell proliferation, differentiation, and immune responses (22, 23). Although TGF-β is recognized as an inhibitor of normal epithelial cell proliferation and an early-stage tumor suppressor, tumor cells often acquire a resistance to the growth-suppressive activities of TGF-β1 during tumor progression (24–26). In some tumors, TGF-β1 expression and activity has been implicated in promoting survival, progression, and metastases (25, 27, 28). This is postulated to be mediated by both paracrine effects in the local tumor-stromal environment, including the effects of TGF-β1 on immune surveillance, angiogenesis, and increased tumor interstitial pressure, as well as autocrine effects inducing survival signals directly in tumor cells (29, 30). Several studies have now shown the antitumor effects of inhibiting the TGF-β1 pathway (24, 27, 28, 31, 32).

We have previously described the generation of potent and selective function blocking $\alpha_v\beta_6$ monoclonal antibodies (mAbs; ref. 33).

Note: L.A. Koopman, D.R. Leone, and S. Ho contributed equally to this work.

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In this article, we further describe the identification of mAbs that specifically bind to denatured β_6, allowing for the analysis of $\alpha_v\beta_6$ expression on paraffin-embedded tissues. Previously generated $\alpha_v\beta_6$ antibodies used for immunohistochemistry have not clearly shown selective binding to the $\alpha_v\beta_6$ integrin. A murine mAb described here, 6.2A1, represents the first $\alpha_v\beta_6$ antibody to be fully characterized, and that shows specific binding to both intact and denatured forms of both human and mouse $\alpha_v\beta_6$ integrin. This is a major advantage in that most human tissues that are available for immunohistochemical analysis are only accessible as paraffin-embedded tissue. The 6.2A1 mAb was used to analyze the expression of $\alpha_v\beta_6$ in a variety of human primary and metastatic cancers, and to compare this expression with that in normal tissue. Further, we generated a human/mouse chimeric version of this mAb (ch6.2A1) to allow immunohistochemical detection of $\alpha_v\beta_6$ on tissue isolated from mouse models. The human/mouse chimeric form of the mAb contains a human IgG1 Fc region, allowing the use of an antihuman secondary antibody. In our studies, squamous carcinomas showed a particularly high frequency of intense $\alpha_v\beta_6$ expression, consistent with a potentially important role for this integrin in both the progression and invasion of oral cancers (34). A function blocking $\alpha_v\beta_6$ mAb, 6.3G9, and rsTGF-βRII-Fc were used to further define the role of $\alpha_v\beta_6$ in squamous cell carcinomas. Both methods of inhibiting TGF-β led to inhibition of Detroit 562 human pharyngeal carcinoma xenograft growth *in vivo*, without a direct inhibitory effect on *in vitro* cell growth. The results described suggest that the *in vivo* growth inhibitory effects may be mediated through both direct autocrine effects on the tumor cells as well as indirect paracrine interactions with the tumor stromal environment.

Materials and Methods

Antibodies and proteins. Murine $\alpha_v\beta_6$ mAbs, 6.3G9 and 6.4B4, used for cell-based assays and *in vivo* efficacy studies, and 6.2A1, used for immunohistochemistry studies, were generated as previously described (33). A human/mouse chimeric form of 6.2A1 (ch6.2A1), used for immunostaining tumor xenograft sections, was generated as described below. Murine rsTGF-βRII-Fc and human soluble $\alpha_v\beta_6$ (hs$\alpha_v\beta_6$) were generated as previously described (33, 35). Human recombinant soluble TGF-β receptor type II (rshuTGF-βRII), LAP1, TGF-β1, and latent TGF-β1 were purchased from R&D Systems. Human plasma fibronectin was purchased from Chemicon. Antibodies were purchased from the following vendors: phycoerythrin-conjugated anti-α_v (Chemicon); goat anti-mouse IgG phycoerythrin- and horse radish peroxidase (HRP)–conjugated anti-rabbit antibody (Jackson ImmunoResearch); donkey anti-goat IgG Alexa 488 and goat anti-rabbit Alexa Fluor 594 (Molecular Probes); goat anti-human TGF-βRII (R&D Systems); biotinylated anti-mouse, anti-goat, and anti-human IgG (Vector Laboratories); rabbit anti-phosphorylated Smad2/3 (p-Smad2/3; Cell Signaling Technology, Inc.); and rabbit anti-TGF-β (Santa Cruz Biotechnology).

Tissue collections. Paraffin-embedded human tissues were obtained from Phenopath, Inc. Tissue arrays were purchased from Imgenex Histoarrays and from Petagen.

Cell lines and cell culture. Detroit 562 human pharyngeal cancer cells were purchased from American Type Tissue Collection Repository and cultured in MEM (Eagle) with Earle's (Invitrogen), 0.1 mmol/L nonessential amino acids (Biowhittaker), 1.0 mmol/L sodium pyruvate (Sigma), 10% fetal bovine serum (FBS; JRH Biosciences), and 2 mmol/L L-glutamine (Mediatech). SW480β6 cells and mink lung epithelial cells transfected with a PAI-1-luciferase reporter gene construct (TMLC) were generated and cultured as previously described (12, 36).

High-performance liquid chromatography purification of β_6 integrin subunit. Purified hs$\alpha_v\beta_6$ was loaded onto a 4.6 mm i.d. × 100 mm POROS R2/H reverse phase high-performance liquid chromatography (HPLC) column (Perseptive Biosystems), using a gradient from 35% to 50% acetonitrile in 0.1% trifluoroacetic acid. Peak fractions containing β_6 were pooled, lyophilized, and reconstituted in PBS. Proteins were analyzed using electrophoresis on a 4% to 20% SDS-polyacrylamide gel (Invitrogen) under nonreducing conditions and visualized using Coomassie blue protein stain.

Generation of human/mouse chimeric, IgG2a, and aglycosylated IgG2a mAbs. ch6.2A1 cDNA was generated from parent hybridoma RNA using a First Strand cDNA synthesis kit (Amersham/Pharmacia). Heavy and light chain variable regions were amplified by PCR using 3′ primers used for cDNA synthesis and pools of degenerate primers and Pfu DNA polymerase (Stratagene). Variable regions were ligated into expression vectors with human IgG1 constant regions.

To generate the IgG2a and aglycosylated IgG2a forms of 6.3G9, DNA encoding the signal sequence and variable region of 6.3G9 was amplified by PCR with oligonucleotide primers containing a 5′ *Not*I and 3′ *Bst*E2 site. Constant regions of wild-type and N300Q (aglycosylated) variant forms of murine IgG2a were PCR amplified from murine IgG2a cDNA with primers containing a 5′ *Bst*E2 and 3′ *Not*I site. Variable and constant regions were cloned into the *Not*I site of the pKJS157 plasmid (Invitrogen). Murine κ chain was generated by PCR amplification of 6.3G9 cDNA with primers containing a 5′ *Xba*I and 3′ *Hin*dIII site. The murine κ constant region was PCR amplified from murine κ cDNA with primers containing a 5′ *Hin*dIII and 3′ *Xba*I site. Light chain variable and constant regions were cloned into the *Xba*I site of the pKJS157 plasmid.

Flow cytometry analysis. Cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (1× PBS, 2% FBS, 0.1% NaN_3, 1 mmol/L $CaCl_2$, and 1 mmol/L $MgCl_2$) containing 6.3G9 or anti-TGF-βRII antibodies, at 10 μg/mL. Where indicated, rshuTGF-βRII was added in 10-fold excess of the TGF-βRII antibody. After incubation for 30 min on ice, cells were washed and resuspended in FACS buffer containing 5 μg/mL phycoerythrin-conjugated secondary antibodies and incubated on ice for 30 min. After washing, binding of secondary antibody was monitored by flow cytometry.

Protein binding assays and cell-based adhesion assays. These assays were carried out as previously described (33).

Proliferation assays. Detroit 562 cells were seeded at 2.5×10^5 per well in 96-well plates in culture medium containing 2% charcoal-stripped serum and allowed to adhere overnight. Active or latent TGF-β1 was added to quadruplicate wells and cells were incubated at 37°C for 24 h for TGF-β1 titration experiments and for 48 h for blocking studies. Latent TGF-β1 was added at 10 ng/mL, and 6.3G9 was added at 10 μg/mL. Methyl-[^{3}H]thymidine (1 μCi; Amersham/GE Healthcare Biosciences) was added per well for the last 4 h of the incubation period. After trypsinization, cells were harvested using a Brandel MXR96TI Harvester (Brandel) on Wallac 1450 filtermats for subsequent measurement of [^{3}H]thymidine incorporation using a Wallac 1450 Microbeta scintillation counter (Wallac/Perkin-Elmer).

TGF-β1 ELISA. Detroit 562 cells were plated at 5×10^6 per well in six-well plates and incubated overnight. Cells were washed with PBS, and culture medium containing 1% FBS medium was added and incubated at 37°C for 24 h. Culture supernatants were collected and analyzed for both active and total TGF-β1 levels using a Quantikine human TGF-β1 immunoassay kit following the protocol provided (R&D Systems).

TGF-β1 bioassay (TMLC coculture assay). This assay was carried out as previously described (33) using Detroit 562 cells and TMLC, mink lung epithelial cells transfected with a PAI-1-luciferase reporter gene construct. mAbs and rsTGF-βRII were assayed at a final concentration of 10 μg/mL.

Immunohistochemistry. Sections were deparaffinized in xylene and ethanol, rehydrated in water, and immersed in methanol containing 0.45% H_2O. Tissues were then incubated with pepsin (Zymed) and blocked with avidin and biotin (Vector Laboratories). For immunostaining β_6 on tumor xenograft tissues, sections were incubated with ch6.2A1 and an antihuman biotinylated secondary antibody. For immunostaining β_6 on human tissue, sections were incubated with 6.2A1 and an antimurine biotinylated secondary antibody. For immunostaining TGF-βRII, sections were incubated with primary antibody, with or without a 10-fold molar excess of rshuTGF-βRII.

Washed sections were incubated with ABC reagent (Vector Laboratories), for 30 min at room temperature, and 3,3′-diaminobenzidine substrate (Vector Laboratories). Frozen tissue sections embedded in OCT compound (Sakura Tokyo) were fixed in acetone and blocked with 0.5% casein/0.05% thimerosal in PBS. For immunostaining, TGF-β sections were incubated with primary antibody and an anti-rabbit Alexa Fluor 488 secondary antibody. Analysis of $\alpha_v\beta_6$ expression used an H-score, which represents a composite score reflecting both the intensity of staining and the percentage of positively stained cells. The H-score represents the sum of the products of the percentage of positive cells (0–100) and the intensity of staining scored on a four-point scale, where 0 represents no staining above background, 1+ represents weak staining, 2+ represents moderate staining, and 3+ represents strong staining. A maximum H-score of 300 represents 100% of cells exhibiting 3+ staining.

Analysis of integrin β_6 and p-Smad2/3 protein expression. For detecting p-Smad2/3, cells were lysed in 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.0), 2 mmol/L EGTA, 2% Triton X-100, and for detecting β_6, cells were lysed in 10 mmol/L HEPES, 0.5 mmol/L $MgCl_2$ (pH 7.5), 1% Triton X-100 each containing Complete Mini protease inhibitor (Roche Diagnostics). After electrophoresis and transfer, membranes were blocked and incubated with 1:10,000 anti-pSmad2/3 or 1 μg/mL 6.2A1, washed, and then incubated with HRP-conjugated secondary antibody. Bands were detected using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Inc.).

Tumor xenograft studies. Female athymic nude mice, 6 to 8 weeks old (Harlan Sprague-Dawley, Inc.) were used for all studies. Mice were maintained in accordance with the Biogen Idec Institutional Animal Care and Use Committee, and city, state, and federal guidelines for the humane treatment and care of laboratory animals. Animals were s.c. implanted (day 0) on the right flank with 5×10^6 Detroit 562 cells, suspended in medium without serum. Tumors were measured with calipers and tumor size (mm) was converted to mg using the formula mg = (length × width ^{2}) / 2. Body weights were monitored throughout the course of each study. Treatments were initiated when the tumor size reached a minimum weight of 80 mg. Studies were run as randomized, double-blinded trials with a vehicle control (n = 30) and treatment groups (n = 10). Antibodies and rsTGF-βRII-Fc were administered i.p. thrice weekly (dosing every other day). No significant effect on body weight was measured for any of the treatment groups. Statistical comparisons were made comparing control groups and test article using the Student's t test.

Results

Expression of $\alpha_v\beta_6$ in squamous cell carcinomas. To characterize the expression of $\alpha_v\beta_6$ across a wide spectrum of human malignant and nonmalignant tissue types, a panel of hybridoma clones was screened for mAbs capable of recognizing the β_6 subunit in immunohistochemical staining of paraffin-embedded tissue sections. To this end, the β_6 subunit was separated chromatographically from hs$\alpha_v\beta_6$ (12) and analyzed by SDS-PAGE and Coomassie blue staining (Fig. 1*A*, *left*). The resultant isolated and denatured β_6 was used for screening hybridoma supernatants for antibody binding using a solid-phase ELISA (data not shown). Murine mAb, 6.2A1, was identified and chosen for further characterization. 6.2A1 specifically recognized β_6 in immunoblots, comparing β_6-transfected SW40 cells with untransfected SW480 cells (Fig. 1*A*, *right*), and also immunostained sections of paraffin-embedded β_6-transfected SW40 cells but not untransfected SW480 cells (Fig. 1*B*). 6.2A1 bound to human $\alpha_v\beta_6$ with a ED_{50} equal to 62.9 ng/mL, and in contrast to our previously described mAbs, 6.2A1 did not inhibit ligand binding to $\alpha_v\beta_6$ (33).

Generation of 6.2A1 allowed for analysis of $\alpha_v\beta_6$ expression across several tumor types by paraffin immunohistochemistry. Although $\alpha_v\beta_6$ was clearly expressed (H-score >50) in a wide spectrum of epithelial malignancies, expression was particularly notable in carcinomas predominantly of squamous cell origin of the cervix, skin, esophagus, and head and neck, which were positive in 92% (n = 46), 84% (n = 49), 68% (n = 56), and 64% (n = 100) of cases, respectively (Table 1). Strong expression of $\alpha_v\beta_6$, defined as an H-score >150, was observed in 63% of cervical, 50% of esophageal, 41% of skin, and 35% of head and neck squamous cell carcinomas. A statistically significant correlation between the level of $\alpha_v\beta_6$ expression and tumor grade and stage was not observed in our studies. An analysis of a larger number of cases, for each tumor type, would be required to determine if a statistically significance relationship exists. In our studies, the n value for most of the individual tumor types was ~50 cases.

The strong expression of $\alpha_v\beta_6$ in malignant tissues, particularly squamous cell carcinoma, sharply contrasts with the absent or minimal expression observed in nonmalignant tissues (Fig. 1*C*, and data not shown). In normal tissue, $\alpha_v\beta_6$ expression was limited to the basal cells of occasional samples of squamous epithelium, in some cases with associated reactive epidermal changes. There was variable but limited expression along the colonic columnar epithelium, by the syncytiotrophoblast cells of placental villi, and in placental membranes (data not shown). $\alpha_v\beta_6$ was not detected on any connective or neural tissue examined (data not shown). Consistent with its restricted expression in epithelium and absence in normal connective tissue, $\alpha_v\beta_6$ was not detected on any sarcomas examined (data not shown).

$\alpha_v\beta_6$ exhibited a unique pattern of expression in a significant percentage of squamous cell carcinomas that was characterized by strong staining of malignant cells located along the periphery of nests of infiltrating tumor tissue (e.g., see Fig. 1*C*). These cells form the interface with the adjacent tumor stroma. Malignant cells located within the tumor focus exhibited reduced, and occasionally absent, staining. This pattern was particularly prominent among squamous cell carcinoma of the head and neck, where ~50% of tumors that were positive for $\alpha_v\beta_6$ expression exhibited this tumor-stroma interface pattern of expression.

Analysis of tumor metastases derived from a wide spectrum of primary epithelial malignancies revealed $\alpha_v\beta_6$ expression in 77% of cases (n = 52; Fig. 1*D*). Among nine cases of primary carcinoma (five cervical, two ovarian, one endometrial, and one thyroid) for which a matched metastasis was available for analysis, all cases exhibited an equivalent or higher level of expression (H-score) in the metastatic lesion. Moreover, the metastatic tumor lesions often exhibited the tumor-stroma interface pattern of $\alpha_v\beta_6$ expression that was commonly observed in primary tumor sections. Together, these results indicate that $\alpha_v\beta_6$ expression persists through a metastatic transition.

***In vitro* characterization of $\alpha_v\beta_6$ function in Detroit 562 cells.** We evaluated the functional properties of $\alpha_v\beta_6$ on the Detroit 562 cell line, derived from a human pharyngeal squamous carcinoma. As determined by flow cytometry, Detroit 562 cells show significant surface expression of $\alpha_v\beta_6$ (Fig. 2*A*). Cell adhesion assays were carried out to determine if these cells adhere to the $\alpha_v\beta_6$ ligands, LAP and fibronectin, and would be inhibited by the function blocking $\alpha_v\beta_6$ mAb, 6.3G9. This mAb has been previously shown to bind selectively to $\alpha_v\beta_6$ with an ED_{50} equal to 14.5 ng/mL (33). Cells bound to both ligands (Fig. 2*B*) and 10 μg/mL of 6.3G9 inhibited adhesion to LAP by >90% and completely inhibited adhesion to fibronectin. Because the expression of $\alpha_v\beta_6$ is not always sufficient to confer the ability to activate TGF-β (13), we tested Detroit 562 cells in a coculture system previously described as a method to monitor $\alpha_v\beta_6$-mediated activation of TGF-β (13).

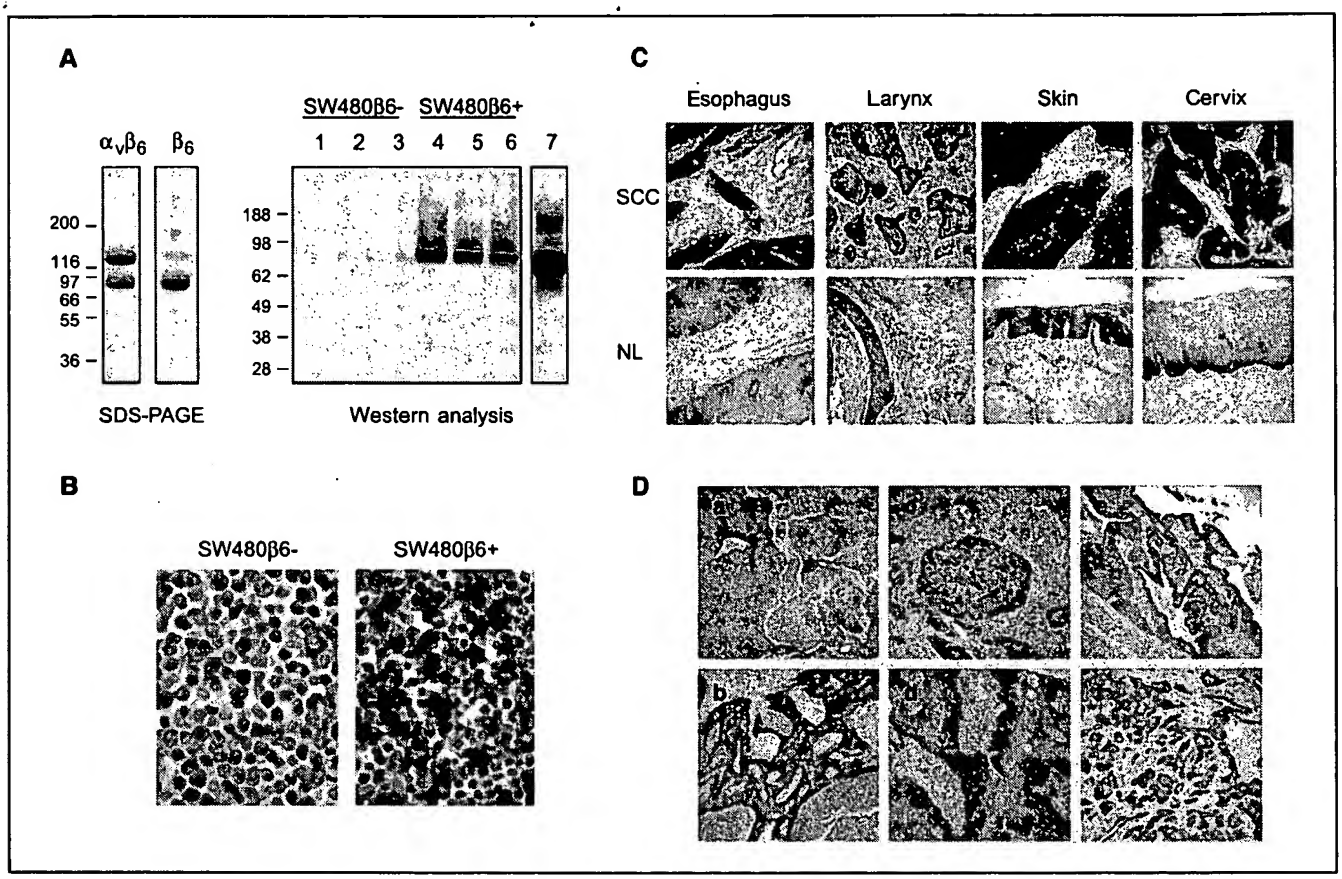

Figure 1. Specificity of 6.2A1 binding to integrin β_6 and immunohistochemical analysis of $\alpha_v\beta_6$ expression in human cancers, metastases, and normal tissue. *A*, the β_6 subunit was purified from hs$\alpha_v\beta_6$ using preparative reverse-phase HPLC, and proteins were analyzed by SDS-PAGE and Coomassie blue staining (*left*). Lysates from SW480β6− and SW480β6+ cells were analyzed by immunoblotting using 6.2A1 (*right*). *Lanes 1* to *3*, SW480β6− lysate; *lanes 5* to *6*, SW480β6+ lysate; and *lane 7*, purified hs$\alpha_v\beta_6$. The band at ~80 kDa represents the β_6 subunit. In lane 7, the higher molecular weight band (~180 kDa) represents a trace amount of SDS-stable $\alpha_v\beta_6$ heterodimer, which was detected using the more sensitive immunoblotting method but was not observed using Coomassie blue staining. *B*, sections of paraffin-embedded SW480β6− and SW480β6+ cell pellets immunostained with 6.2A1. SW480β6+ cells represent a nonclonal cell line and thus expression of $\alpha_v\beta_6$ is variable within the cell pellet. Magnification, ×400. *C*, immunohistochemical analysis of $\alpha_v\beta_6$ expression in human cancers and normal tissue. Representative examples of strong $\alpha_v\beta_6$ expression in squamous cell carcinoma (*SCC*) and low-level expression in normal (*NL*) tissue from the indicated anatomic sites. Section of normal tissue from larynx represents nonmalignant tissue adjacent to a site of primary squamous cell carcinoma and exhibits mild inflammation with reactive changes. Sections of normal and malignant tissues from each site were present on the same tissue microarray (with the exception of esophagus), thus providing identical assay conditions for comparison of expression in normal versus malignant tissue. All images were obtained at ×200 magnification using identical digital image capture variables. *D*, $\alpha_v\beta_6$ expression in metastatic tumor lesions. Metastases to lymph node (*a–d*) and lung (*e* and *f*) are shown from primary squamous cell carcinomas of the cervix (*a*), esophagus (*b*), skin (*c*), and head and neck (*d–f*). The specific sites of the primary head and neck malignancies include tongue (*d*), larynx (*e*), and neck (not otherwise specified; *f*). All images at ×200 magnification.

Coculture of Detroit 562 cells with TMLC led to a significant increase in luciferase activity relative to coculture with negative control SW480 cells (Fig. 2*C*). 6.3G9 and rsTGF-βRII-Fc were tested at 10 μg/mL for their ability to block Detroit 562–mediated TGF-β activation. 6.3G9 reduced activation to background level, whereas rsTGF-βRII-Fc inhibited activation by 44% (Fig. 2*C*). The cells were assayed for their ability to secrete TGF-β1. Active TGF-β1 was barely detectable whereas ~300 pg/mL of acid activated latent TGF-β1 were produced in culture after 24 h as measured by ELISA (Fig. 2*D*). These findings suggest that Detroit 562 cells produce latent TGF-β1 that can be activated in an $\alpha_v\beta_6$–dependent manner.

Effects of TGF-β, $\alpha_v\beta_6$ mAbs, and rsTGF-βRII-Fc on Detroit 562 cellular proliferation and Smad phosphorylation *in vitro*. To determine whether the TGF-β signaling pathway is intact in Detroit 562 cells, we confirmed the expression of TGF-βRI, TGF-βRII, and Smad4 protein, and evaluated the phosphorylation status of Smad2/3 before and after treatment with latent and active forms of TGF-β1. TGF-βRI and Smad4 were expressed by Detroit 562 cells as determined by Western blot analysis (data not shown). TGF-βRII was also expressed as determined by flow cytometry and immunohistochemical analysis of xenograft sections (Fig. 3*A* and *B*). The binding of anti–TGF-βRII antibody was completely inhibited by the coincubation of rshuTGF-βRII (Fig. 3*A* and *B*). TGF-βRI and TGF-βRII contain no mutations in Detroit 562 cells (data not shown and ref. 37). To confirm that the TGF-β1 receptors are capable of mediating intracellular signaling, we analyzed p-Smad2/3 in the cells in response to the addition of TGF-β1 (Fig. 3*C*). Addition of either the active or latent forms of TGF-β1 induced p-Smad2/3. In keeping with the ability of $\alpha_v\beta_6$ to activate TGF-β, we analyzed the effect of 6.3G9 on the phosphorylation of Smad2/3 in Detroit 562 cells alone or in combination with latent or active TGF-β1. The addition of 6.3G9 significantly reduced both the basal level of p-Smad2/3 and the phosphorylation induced by the addition of 100 ng/mL latent TGF-β1 (Fig. 3*C*). As expected, 6.3G9 had no effect on p-Smad2/3

Table 1. $\alpha_v\beta_6$ expression in human cancer

Tumor site	Positive expression (H-score >50), %	N
Cervix	92	46
Skin	84	49
Esophagus	68	56
Head and neck	64	100
Breast	43	145
Lung*	35	54
Ovarian	33	52
Kidney	21	103
Colorectal	12	60
Hepatocellular	<2	59

*There was no significant difference in the frequency of positive expression in cases of squamous cell carcinoma of the lung compared with nonsquamous cell carcinoma.

induced by the addition of active TGF-β1 because the LAP portion of the molecule is not present. The addition of rsTGF-βRII-Fc inhibited both the basal level of p-Smad2/3 and that induced by the addition of active TGF-β1.

We also tested the effects of TGF-β1 (38) on the proliferation of Detroit 562 cells *in vitro*. The addition of either the active (Fig. 4*A*) or latent form of TGF-β1 (Fig. 4*B*) led to a modest inhibition of growth. A maximum inhibition of 20% was achieved by active TGF-β1 that was statistically significant at concentrations ≥74 pg/mL ($P < 0.05$). A maximum inhibition of 25% was achieved with latent TGF-β1 that was statistically significant at concentrations ≥195 pg/mL ($P < 0.05$). The addition of 6.3G9 or rsTGF-βRII-Fc had no direct effects on the proliferation of Detroit 562 cells (Fig. 4*C*) nor did 6.3G9 block the inhibition of growth induced by the addition of latent TGF-β1 (Fig. 4*D*). In similar studies, Detroit 562 cells were cultured in collagen or Matrigel and no significant effects of 6.3G9 on cell proliferation were observed (data not shown). Collectively, these findings suggest that whereas $\alpha_v\beta_6$ can mediate TGF-β–induced intracellular signaling, it does not seem to regulate Detroit 562 proliferation in an autocrine manner as assessed in this *in vitro* system.

Effect of anti-$\alpha_v\beta_6$ mAbs and rsTGF-βRII-Fc on growth of Detroit 562 cells *in vivo*. Detroit 562 cells grown as xenograft tumors were evaluated for the expression of $\alpha_v\beta_6$ and TGF-β using immunohistochemistry. Although the majority of cells express $\alpha_v\beta_6$ by flow cytometry, the xenograft tumors were found to express $\alpha_v\beta_6$ predominantly at the tumor-stroma interface (Fig. 5*A*). This suggests that there is further regulation of $\alpha_v\beta_6$ expression *in vivo*

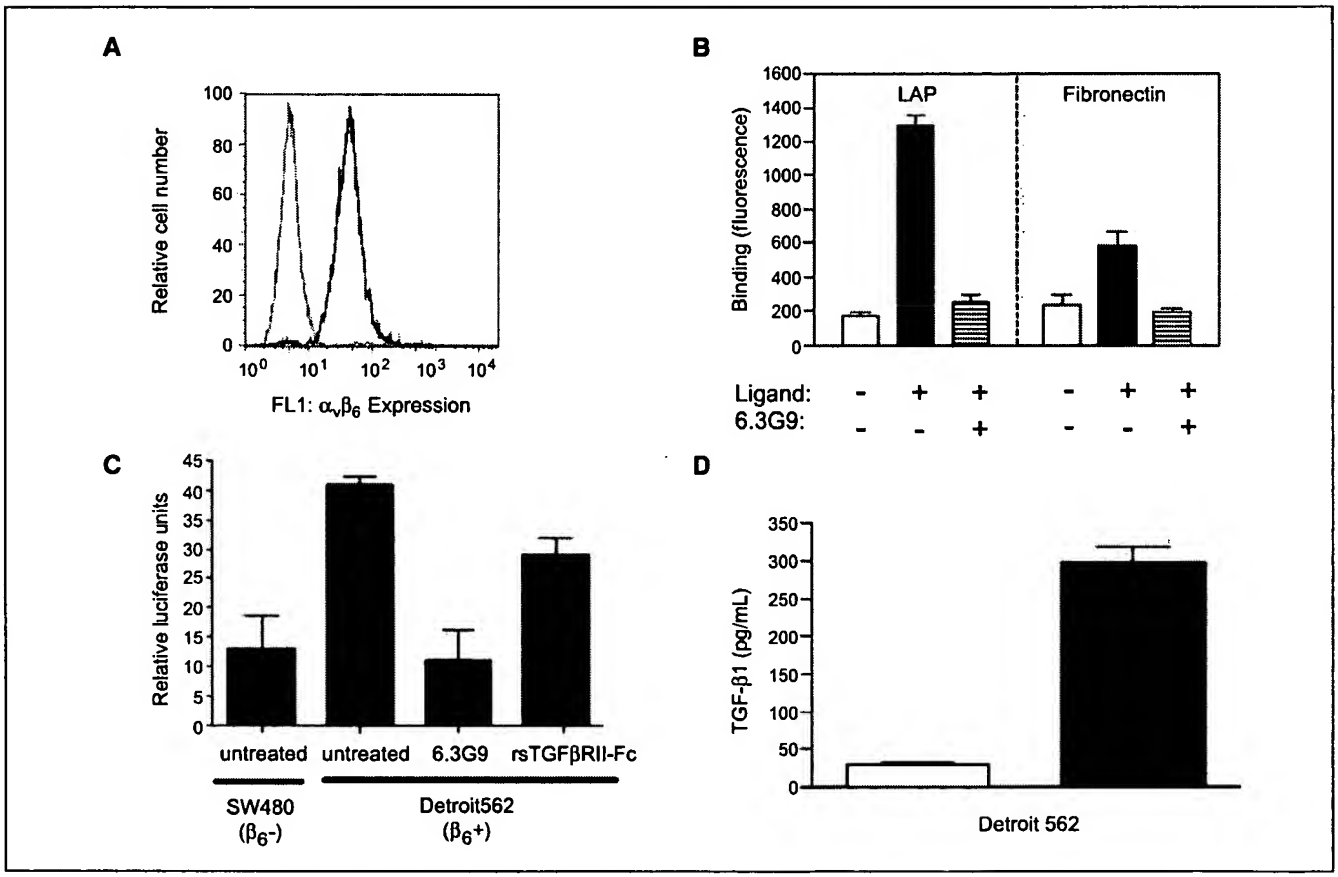

Figure 2. Detroit 562 cell line characterization. *In vitro* assays were performed to study $\alpha_v\beta_6$ expression and function in Detroit 562 cells. *A*, flow cytometry analysis of $\alpha_v\beta_6$ expression (*black line*, 6.3G9; *gray line*, secondary antibody only). *B*, cell adhesion to LAP or fibronectin in the presence or absence of 10 μg/mL 6.3G9. $P < 0.0001$, 6.3G9 versus untreated in LAP adhesion assay. $P = 0.0001$, 6.3G9 versus untreated in fibronectin adhesion assay. *C*, TGF-β activation assay of Detroit 562 cells cocultured with TMLC, with or without 10 μg/mL 6.3G9 or rsTGF-βRII-Fc. SW480 cells (β_6-negative cells) were included as a control for background luciferase activity. $P = 0.005$, 6.3G9 versus untreated and $P = 0.0146$, rsTGF-βRII-Fc versus untreated. *D*, production of active (*open columns*) and total acid activated TGF-β1 (*closed columns*). *Columns*, mean from a representative experiment containing triplicate samples; *bars*, SD.

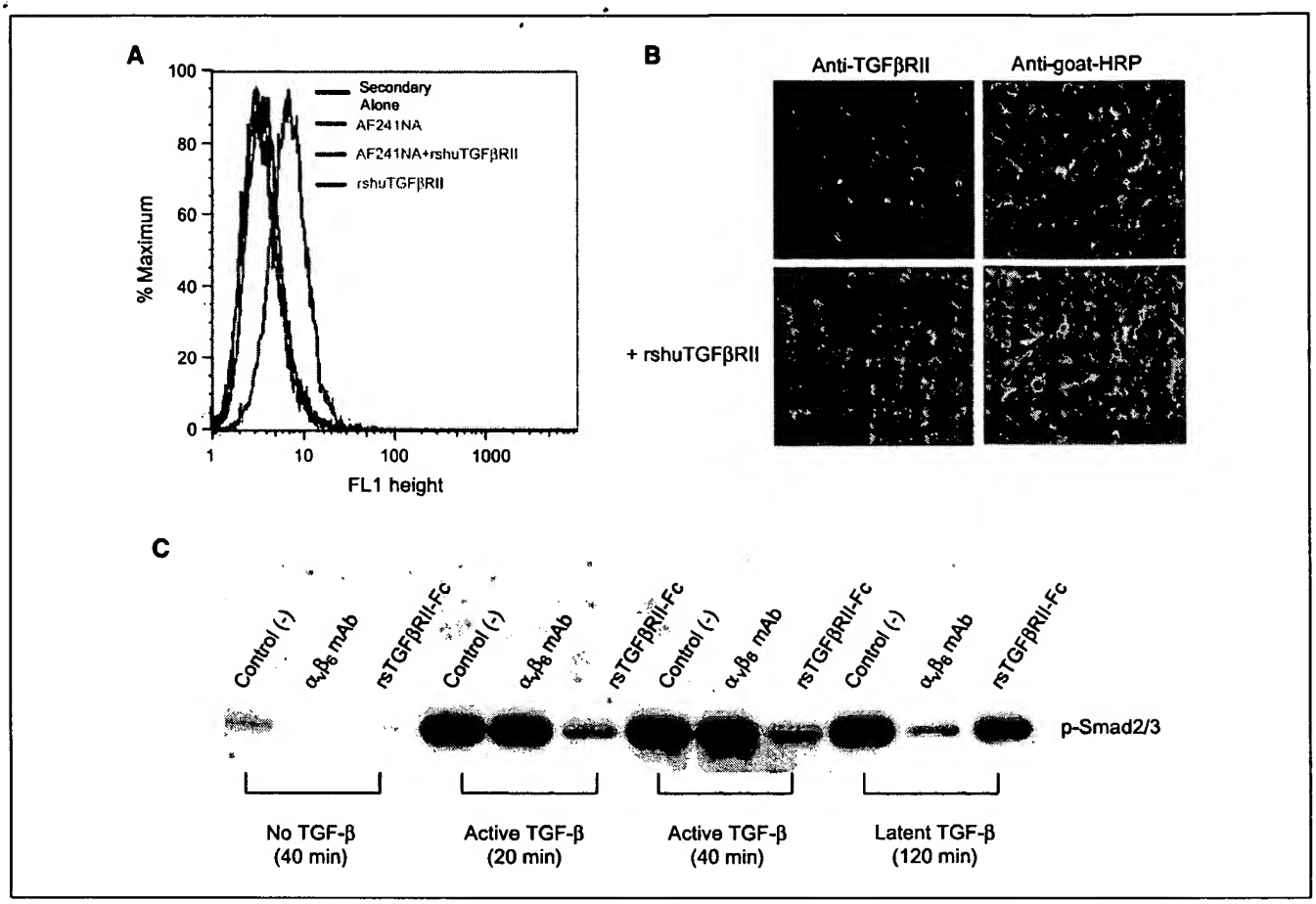

Figure 3. Analysis of TGF-βRII expression and Smad2/3 phosphorylation in Detroit 562 cells. *A*, flow cytometry analysis of TGF-βRII antibody binding to Detroit 562 cells with or without co-addition of rshuTGF-βRII. *B*, immunohistochemical analysis of TGF-βRII antibody binding to Detroit 562 xenograft sections with or without co-addition of rshuTGF-βRII. Magnification, ×400. *C*, immunoblot of phosphorylated Smad2/3 in Detroit 562 cells. Cells were serum starved for 3 h in DMEM and then treated with active or latent active TGF-β1 alone or in combination with 6.3G9 or rsTGF-βRII-Fc for the indicated times. Proteins were tested at the following concentrations: 2 ng/mL active TGF-β1, 100 ng/mL latent TGF-β1, 10 μg/mL 6.3G9, and 10 μg/mL rsTGF-βRII-Fc.

leading to increased expression at the tumor-stromal interface. Immunohistochemical analysis of TGF-β in the xenograft sections showed significant TGF-β in the surrounding stroma (Fig. 5*A*). We investigated whether treatment with $\alpha_v\beta_6$ mAbs would inhibit tumor growth and also tested the effect of rsTGF-βRII-Fc as an inhibitor of the TGF-β pathway. Treatments included function blocking $\alpha_v\beta_6$ mAb, 6.3G9; non–function blocking $\alpha_v\beta_6$ mAb, 6.4B4; rsTGF-βRII-Fc; or vehicle control (Fig. 5*B–D*). In a dose ranging study, 6.3G9 inhibited tumor growth in a dose-dependent manner resulting in 50% inhibition at 10 mg/kg compared with vehicle control–treated mice ($n = 10$, $P < 0.05$; Fig. 5*B*). Equivalent doses of 6.4B4, an antibody that binds with high affinity to $\alpha_v\beta_6$ but shows minimal ligand blocking activity (33), had no effect on tumor growth (Fig. 5*C*). rsTGF-βRII-Fc, at 2 mg/kg, resulted in a nearly complete inhibition of tumor growth ($n = 10$, $P < 0.001$; Fig. 5*B* and *C*). In four separate experiments, 6.3G9 produced a statistically significant inhibition of tumor growth with activity ranging from 40% to 70% compared with vehicle control. Inhibition *in vivo* by a function blocking $\alpha_v\beta_6$ mAb and rsTGF-βRII-Fc, and not by the nonblocking $\alpha_v\beta_6$ mAb, suggests that blocking TGF-β activity is the relevant mechanism of action of these molecules.

Additional xenograft studies were conducted to evaluate the importance of antibody-mediated effector function for 6.3G9. An IgG2a version of 6.3G9 was generated to provide optimal effector function in mice. In addition, an IgG2a construct with an N300Q mutation in the CH2 domain of the mAb was generated to eliminate glycosylation of the mAb and to inhibit Fcγ receptor binding as previously reported (39, 40). Similar approaches have been taken with other mAbs to evaluate the role of effector function *in vivo* (41). Wild-type and mutant aglycosylated IgG2a versions of 6.3G9 showed similar binding affinities for $\alpha_v\beta_6$ and blocked ligand binding with similar potencies (data not shown). All forms of the mAb had a similar serum half-life *in vivo* (data not shown). Wild-type and aglycosylated IgG2a forms of 6.3G9 produced similar inhibitory effects, which was comparable with the inhibition of growth detected with the parent IgG1 version of 6.3G9 (Fig. 5*D*). These findings indicate that the *in vivo* efficacy detected with 6.3G9 is not mediated by effector function.

Discussion

The expression of $\alpha_v\beta_6$ in a variety of epithelial malignancies was analyzed using the 6.2A1 antibody. These studies showed that $\alpha_v\beta_6$ was most highly up-regulated in squamous cell carcinomas, where it was found to be expressed in more than 60% of cases evaluated of the head and neck, esophagus, cervix, and skin, thus further

validating and extending previous reports of $\alpha_v\beta_6$ in human carcinomas (6, 9–11, 42). In addition, 77% of metastatic lesions, representing a range of tumor types that had metastasized to different tissues, were found to overexpress $\alpha_v\beta_6$. An association of $\alpha_v\beta_6$ expression with clinical variables such as disease progression, metastases, and survival has been described (9, 11, 42, 43). However, although a correlation between $\alpha_v\beta_6$ expression and tumor grade and stage has been reported in cervical squamous cell carcinomas (11), no correlation was found to exist in other studies analyzing different tumor types (42, 44, 45). These differences may relate in part to the sample size evaluated or reflect real differences among the various tumor types. Collectively, these findings support the hypothesis that $\alpha_v\beta_6$ may facilitate tumor progression, invasion, and metastasis, and may represent a prognostic marker in certain tumor types.

We observed increased expression of $\alpha_v\beta_6$ on head and neck carcinomas particularly at the invasive edge where tumor cells are in direct contact with the tumor-associated stroma. Similar leading edge expression was found in the Detroit 562 xenograft tumors, thus offering an attractive model to study functional effects of blocking $\alpha_v\beta_6$ *in vivo*. *In vitro*, the function blocking $\alpha_v\beta_6$ mAb, 6.3G9, inhibited latent TGF-β–induced Smad2/3 phosphorylation in Detroit 562 cells, but had no direct effect on proliferation. However, *in vivo*, both 6.3G9 and rsTGF-βRII-Fc significantly inhibited Detroit 562 tumor growth. Because the inhibitory effects of mAbs on tumor growth *in vivo* can be mediated at least in part by antibody-dependent cellular cytotoxicity (41, 46), we generated an IgG2a version of 6.3G9 with an N300Q mutation in the CH2 domain of the mAb. Glycosylation of this domain mediates Fcγ receptor binding and is inhibited by this mutation, subsequently attenuating antibody-dependent cellular cytotoxicity (39, 40). These studies revealed that the ability of 6.3G9 to inhibit tumor growth *in vivo* was not related to effector function as the wild-type and mutant forms of the antibody had equivalent effects on tumor growth. The difference in the degree of tumor growth inhibition observed comparing 6.3G9 and rsTGF-βRII-Fc may reflect the extent of TGF-β inhibition by the different therapeutics. rsTGF-βRII has the potential to inhibit all active TGF-β present in the stromal compartment, whereas 6.3G9 is limited to inhibition of TGF-β activation at the epithelial tumor-stromal interface.

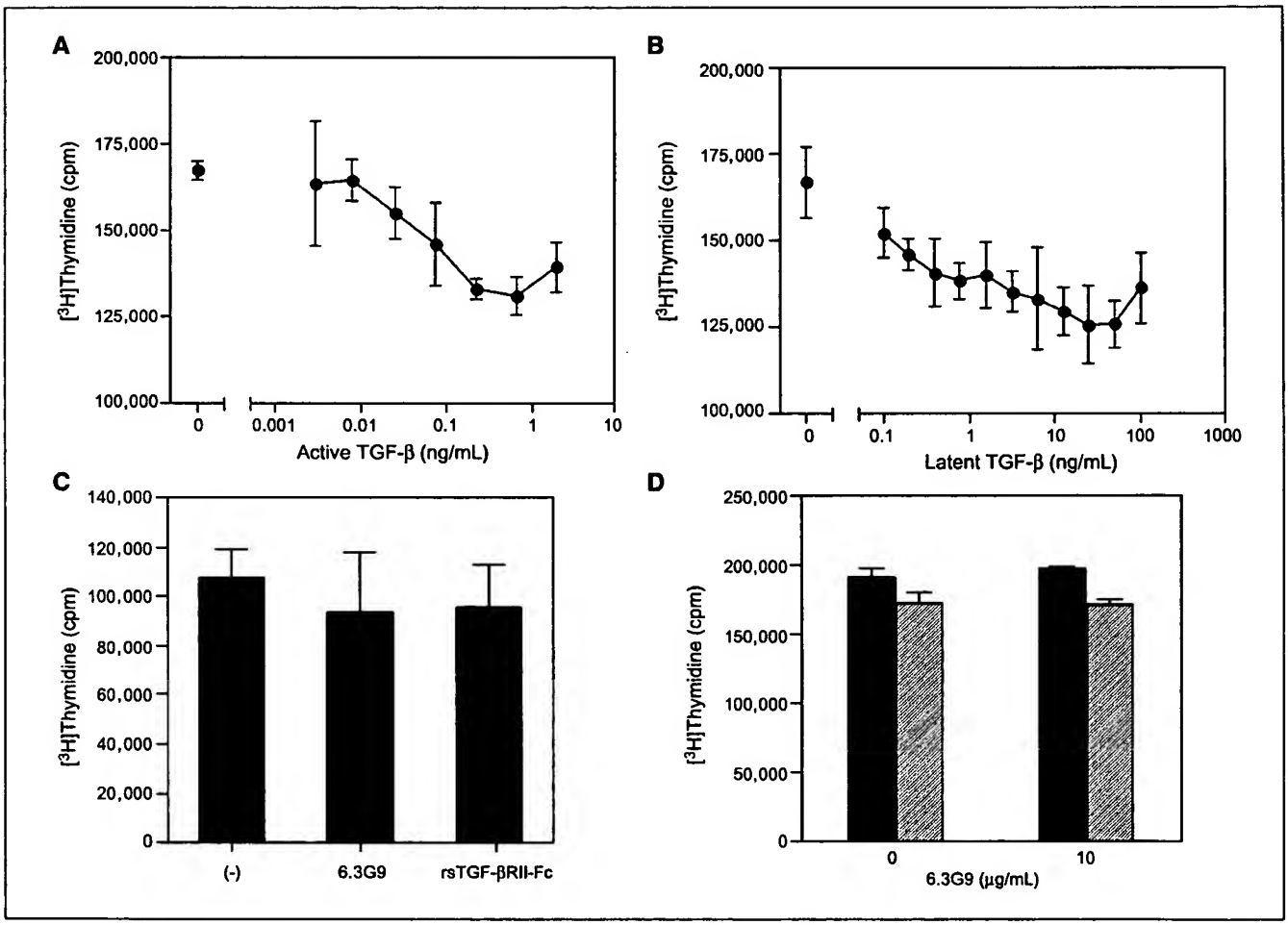

Figure 4. Effects of TGF-β1 and 6.3G9 on Detroit 562 cell proliferation *in vitro*. *A*, dose response of active TGF-β1. All doses of active TGF-β1 at concentrations ≥74 pg/mL had a *P* value <0.05. *B*, dose response of latent TGF-β1. All doses of latent TGF-β1 at concentrations ≥195 pg/mL had a *P* value <0.02. *C*, effects of 6.3G9 or rsTGF-βRII-Fc (10 μg/mL). No significant difference was detected comparing treatments to control. *D*, effect of 10 μg/mL 6.3G9 alone (*solid columns*) or in combination with 10 ng/mL latent TGF-β1 (*hatched columns*). No significant difference was detected with 6.3G9 treatment. Proliferation was measured as [^{3}H]thymidine incorporation into cells.

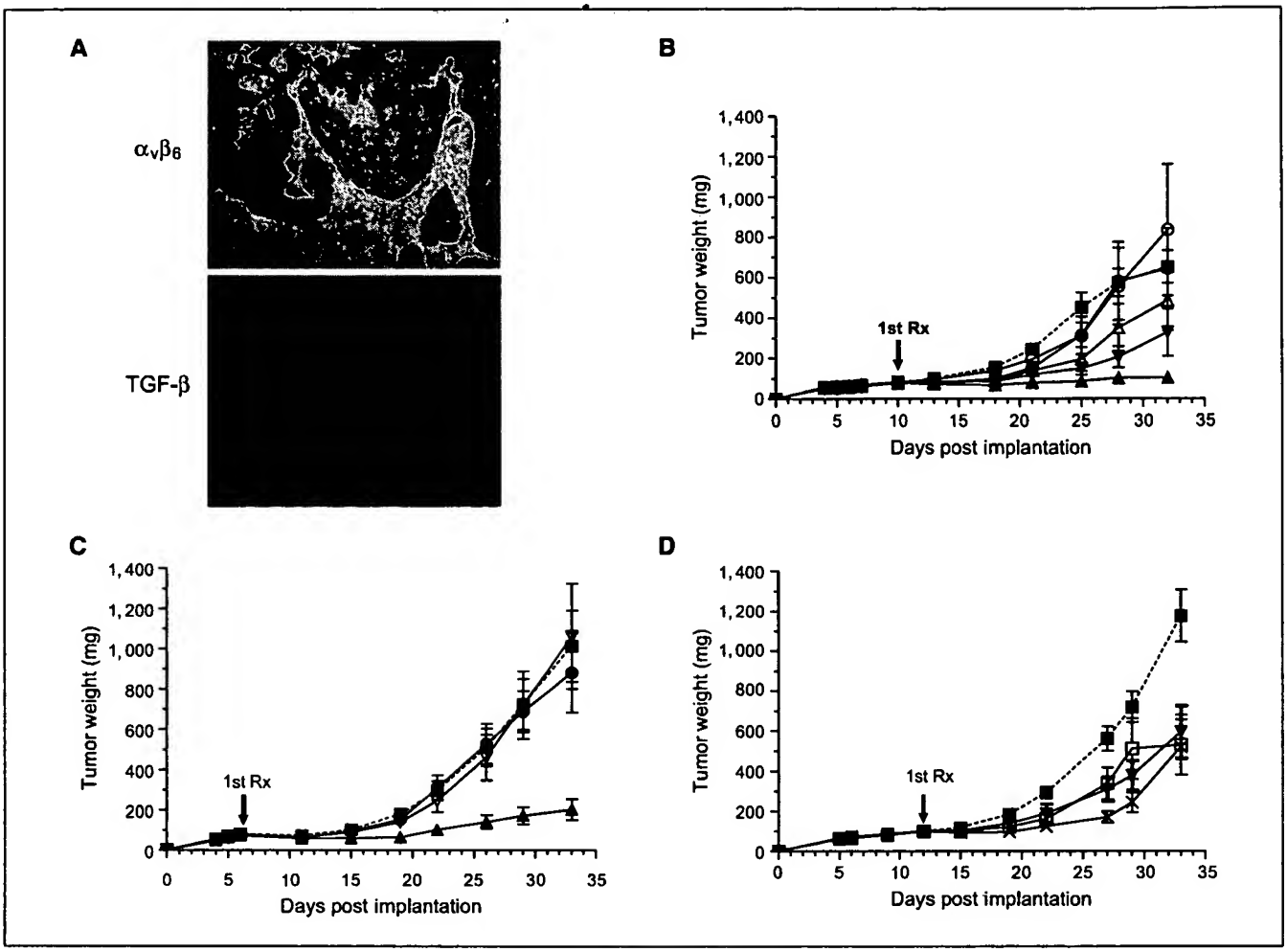

Figure 5. Expression of $\alpha_v\beta_6$ and TGF-β in Detroit 562 xenografts and effects of $\alpha_v\beta_6$ mAbs and rsTGF-β1RII-Fc on Detroit 562 xenograft tumor growth *in vivo*. *A*, Detroit 562 xenograft tissue sections immunostained for $\alpha_v\beta_6$ and TGF-β. Magnification, ×100. *B* to *D*, effects of $\alpha_v\beta_6$ mAbs and rsTGF-βRII-Fc on xenograft tumor growth. *B*, dose response of 6.3G9. Vehicle (■); 10 mg/kg 6.3G9 (▼), $P < 0.01$ days 18, 21, 25, and 32; $P < 0.05$ day 28; 4 mg/kg 6.3G9 (△), $P < 0.05$ days 18, 21, 28, 32, and $P > 0.05$ day 25; 1 mg/kg 6.3G9 (●), $P < 0.05$ day 18 and no significant difference at days 21 to 32; 4 mg/kg 6.4B4 (○), no significant difference at any time point; and 2 mg/kg rsTGF-βRII-Fc (▲), $P < 0.001$ days 18 to 32. All comparisons were made relative to vehicle control group. *C*, dose response of 6.4B4. Vehicle (■); 4 mg/kg 6.4B4 (●), no significant difference at any time point; 10 mg/kg 6.4B4 (▽) no significant difference at any time point; and 2 mg/kg rsTGF-βRII-Fc (▲), $P < 0.05$ days 11, 22, and $P < 0.01$ days 15, 19, 29, and 33. All comparisons were made relative to vehicle control group. *D*, effect of different Ig forms of 6.3G9. Vehicle (■); 10 mg/kg 6.3G9 IgG1 (▼), $P < 0.05$ days 27 to 33; 10 mg/kg 6.3G9 IgG2a (□), $P < 0.05$ days 19, 22, 27, 33, and >0.05 day 29; and 10 mg/kg 6.3G9 aglycosylated IgG2a (× mark), $P < 0.05$ days 19 to 33. All comparisons were made relative to vehicle control group. *Arrows*, time of initiating dosing.

The $\alpha_v\beta_6$ integrin not only mediates TGF-β activation, but has also been reported to regulate intracellular signaling upon binding to extracellular matrix ligands. *In vitro* and *in vivo* studies have suggested that $\alpha_v\beta_6$ binding to fibronectin can promote migration, invasion, matrix metalloproteinase production, and metastases through fyn and extracellular signal-regulated kinase/mitogen-activated protein kinase (MAPK) signaling pathways (16, 34, 47). Our studies evaluating the role of $\alpha_v\beta_6$ mediating intracellular signaling through MAPK and AKT did not reveal direct effects of blocking $\alpha_v\beta_6$ with mAbs on these downstream events in Detroit 562 cells *in vitro* (data not shown). Our findings suggest that the *in vivo* tumor inhibitory effects of blocking $\alpha_v\beta_6$ or TGF-β may be mediated in part through paracrine effects involving the tumor microenvironment. The predominant expression of $\alpha_v\beta_6$ on the leading edge of tumors suggests that expression may be mediated by matrix interactions. *In vitro* culture of Detroit 562 cells in the presence of collagen I, collagen IV, laminin, or fibronectin resulted in no difference in $\alpha_v\beta_6$ expression as determined by flow cytometry (data not shown). However, immunostaining of Detroit 562 xenograft tissue for smooth muscle actin and collagen (Sirius red stain) consistently exhibited a more reticular pattern of expression along the periphery of the xenograft tumor (data not shown), similar to the localized expression of $\alpha_v\beta_6$ on the leading edges of the same tumors. These observations are consistent with a correlation between $\alpha_v\beta_6$ expression and spatial characteristics of the associated matrix. Similarly, staining of human oral carcinoma revealed smooth muscle actin staining on the invasive front of the tumors at the same locations where $\alpha_v\beta_6$ expression was detected. Although we observed this pattern of expression in both xenografts and human primary tumors, no clear differences in collagen and smooth muscle actin staining was observed in the xenograft studies comparing the different treatment groups. Additionally, no difference in angiogenesis, as determined by CD31 immunostaining, was detected (data not shown). It is possible that $\alpha_v\beta_6$ plays a role

in tumor progression *in vivo* through its proinvasion properties (17, 20, 21). The ability of $\alpha_v\beta_6$ to promote tumor cell invasion into the surrounding stroma may induce a growth-permissive environment. We found that Detroit 562 cells invade *in vitro* and this activity is blocked by the addition of $\alpha_v\beta_6$ mAbs (data not shown). It is possible that *in vivo*, the $\alpha_v\beta_6$ blocking mAb is affecting tumor progression through direct effects on the invasive properties of the tumor cells. This would be consistent with the predominant expression of $\alpha_v\beta_6$ on the leading invasive edge of the Detroit 562 tumor xenografts. It has been shown that TGF-β can promote increased expression of $\alpha_v\beta_6$ (7). Consequently, $\alpha_v\beta_6$ expressed on tumors provides a mechanism for local activation of TGF-β, which can, in turn, further drive increased $\alpha_v\beta_6$ expression *in vivo*. This could create a self-sustaining positive-feedback loop leading to maintenance of TGF-β activation in a localized manner. In support of this concept, we found that expression of $\alpha_v\beta_6$ on Detroit 562 xenograft tumors was nearly completely down-modulated by *in vivo* treatment with rsTGF-βRII-Fc (data not shown). In the xenograft model, it is possible that 6.3G9 disrupts a feedback loop by inhibiting TGF-β activation, and rsTGF-βRII-Fc could do so by blocking TGF-β directly.

$\alpha_v\beta_6$ expression is minimal to negligible in most normal tissues but highly up-regulated on epithelial tumors and metastases. Thus, $\alpha_v\beta_6$ mAbs provide an approach for localized suppression of TGF-β, which could otherwise be a major limitation to the use of systemic inhibitors of the TGF-β pathway. The importance of this is underscored by the known homeostatic roles of the TGF-β pathway in regulating inflammation, immune tolerance, and tumor suppression (22, 23, 48, 49). A comparison of the genetic null phenotypes for $\alpha_v\beta_6$ and TGF-β, and conditional TGF-β pathway null mice, further supports the hypothesis that blocking $\alpha_v\beta_6$-mediated TGF-β activation may be preferable to global targeting of the TGF-β pathway in disease. Mice completely deficient for $\alpha_v\beta_6$ function develop normally and have a typical lifespan, with mild inflammation that is limited to the lung and skin, and with late-onset emphysema (50). In contrast, TGF-β–deficient mice that do not die *in utero* show severe inflammation in multiple organ systems, resulting in death at 3 to 4 weeks of age, and conditional TGF-β pathway null mice have been associated with significantly increased tumorigenesis (23, 48, 49, 51, 52). Thus, $\alpha_v\beta_6$ blocking mAbs provide an opportunity to inhibit tumor growth and minimize the potential for tumor promoting and pro-inflammatory activity that could be associated with systemic blockade of TGF-β.

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